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A Sensitive Time-Resolved Immunofluorometric Assay for the Measurement of Apolipoprotein B in Cerebrospinal Fluid. Application to Multiple Sclerosis and Other Neurological Diseases

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Summary: Although low density lipoprotein receptors have been described on oligodendrocytes, apolipoprotein B was thought to be absent or present in only very small amounts in cerebrospinal fluid (CSF). Several immunoassays have been used for the measurement of apolipoprotein B in serum. However, the majority of methods cannot be used to measure small amounts of apolipoprotein B in CSF. In this study, we describe a highly sensitive time resolved immunofluorometric assay (TR-IFMA) using europium as label (detection limit: 0,3 µg/l). The reliability of the TR-IFMA for the measurement of apolipoprotein B was first studied in serum. Serum and CSF apolipoprotein B concentrations were then determined in subjects free of neurological disorders and in patients with multiple sclerosis. Local intrathecal apolipoprotein B synthesis was calculated. Although the high sensitivity of the TR-IFMA allowed low amounts of apolipoprotein B in CSF to be detected (0.11 ± 0.06; 0.12 ± 0.06 mg/l in controls and multiple sclerosis patients, respectively), no apolipoprotein B could be detected in CSF by electroimmunodiffusion. As suggested by the blood/CSF apolipoprotein B ratio (about 6000), no apolipoprotein B synthesis was observed by both using apolipoprotein B index and formula. This indicates its probable serum origin. Moreover, there was no difference between controls and multiple sclerosis patients in CSF, serum, blood/CSF, index, and local intrathecal apolipoprotein B synthesis. Finally, these results suggest that the role of apolipoprotein B in lipid transport in the central nervous system may be questionable.

Introduction

The central nervous system, together with peripheral neural tissue, possesses a lipid transport system involving apolipoproteins which is responsible for maintaining cholesterol and lipid homeostasis (1). Multiple sclerosis is a neurological disease characterized by primary destruction of myelin and followed by marked alterations of myelin cholesterol and lipid metabolism (2). Apolipoprotein B is involved in the mediation of lipoprotein binding, uptake and cholesterol delivery via low density lipoprotein (LDL) receptors. Although LDL receptors have been described on oligodendrocytes (1), apolipoprotein B was thought to be absent (1, 3) or present in only very small amounts in cerebrospinal fluid (CSF) (4) with the exception of cerebrotendinous xanthomatosis (5).

Most immunoassays have been used for the measurement of apolipoprotein B in serum (6–9). However, the most suitable methods, immunoturbidimetric assay and immunonephelometric assay, cannot be used to measure very small amounts of apolipoprotein B in CSF. Enzyme linked immunosorbent assays (ELISA) and radioimmunoassay (RIA) are sensitive, but the linear working range of the curve of ELISA is small (10, 11) and RIA requires a radiolabelling license (12). To measure apolipoprotein B in CSF, we have developed a very sensitive

method: time-resolved immunofluorometric assay (TR-IFMA) using europium as label. The value of TR-IFMA for the measurement of proteins present in small amounts in CSF has been reported elsewhere (13–15). The reliability of TR-IFMA for the measurement of apolipoprotein B was first studied in serum. A comparative study was then performed with an electroimmunodiffusion on serum and CSF samples. Because usual techniques are not sensitive enough to measure small amounts of apolipoprotein B, a comparative study was performed immunonephelometrically with serum samples from normolipidaemic and dyslipidaemic subjects. CSF and serum apolipoprotein B concentrations were then determined in subjects free of neurological disorders and in patients with multiple sclerosis. Local intrathecal apolipoprotein B synthesis was calculated.

Materials and Methods

Study population

Three groups of patients were studied

The first group (group A) consisted of 24 controls (7 women, 17 men, average age: 42 ± 13 years, range: 22–63) to determine the reference range of apolipoprotein B in CSF. These samples were then used in a comparison between TR-IFMA and electroimmunodiffusion. None of the controls had an increased immunoglobulin G (IgG) index or an oligoclonal band in their CSF, and none had evidence of active demyelinating disease. The integrity of the blood-brain barrier was assessed by measuring albuminorachia (serum albumin: 43164 ± 5466 mg/l; CSF albumin: 228.5 ± 78.4 mg/l). None of the controls had dyslipoproteinaemia (cholesterol: 4.65 ± 1.98 mmol/l; triacylglycerols: 1.56 ± 0.37 mmol/l).

The second group (group B) was composed of 30 multiple sclerosis patients (24 women, 6 men, average age: 39 ± 15, range: 29–65) with an increased IgG index and oligoclonal banding in CSF. None had important blood-CSF barrier damage (serum albumin: 42530 ± 6221 mg/l; CSF albumin: 250.0 ± 98.0 mg/l) or dyslipoproteinaemia (cholesterol: 4.80 ± 0.2 mmol/l; triacylglycerols: 1.76 ± 0.23 mmol/l).

Paired serum and CSF samples were collected during a single 24 h period and stored at −70 °C until analysis.

The third group (group C) consisted of 94 subjects, whose sera were used for an intermethod comparison of apolipoprotein B values in serum between TR-IFMA and immunonephelometry. Thirty-seven subjects with a mean (± standard deviation) cholesterol concentration of 5.18 ± 0.63 mmol/l (range: 4.10–5.90 mmol/l) and triacylglycerol concentrations of 1.02 ± 0.29 mmol/l (range: 0.48–1.35 mmol/l) formed the normolipidaemic subgroup. Twenty-five subjects with mean cholesterol concentrations of 7.49 ± 1.10 mmol/l (range: 6.20–10.80 mmol/l) and triacylglycerol concentrations of 1.10 ± 0.21 mmol/l (range: 0.71–1.40 mmol/l) formed the hypercholesterolaemic subgroup. Thirty-two subjects with a mean cholesterol concentration of 5.15 ± 0.94 mmol/l (range: 2.80 ± 6.50 mmol/l) and triacylglycerolaemia of 3.02 ± 1.36 mmol/l (range: 1.61–7.36 mmol/l) formed the hypertriacylglycerolaemic subgroup. Sera were stored at 4 °C for 24 h until being assayed.

Measurement of apolipoprotein B by TR-IFMA

Apparatus

We used a time-resolved fluorometer (1230 Arcus™), a 12-well aspirating-washing device (1296-024 Platewash), and an automated shaker (1296-001 Plateshake), all from Pharmacia (Uppsala, Sweden).

Reagents

We used a purified rabbit anti-human apolipoprotein B IgG antibody (Interservice B. M., Brazzaville, Bénin) in phosphate buffer (0.1 mol/l, pH 7.4). Delfia™ europium-labeling kits were purchased from Pharmacia. Each kit contains 0.2 mg of labeling reagent (N^1-(*p*-isothiocyanatobenzyl)-diethylenetriamine-N^1,N^2,N^3,N^3-tetraacetate-europium), a 100 nmol/l europium standard, highly purified bovine serum albumin (5 g/l in Tris-HCl 50 mmol/l, pH 7.8, NaN_3, 0.5 g/l), stabilizer, and enhancement solution (per litre, 15 mmol 2-naphthoyltrifluoroacetone, 50 mmol tri-*n*-octylphosphine oxide, 100 mmol acetic acid, 6.8 mmol potassium hydrogen phthalate, and 1.0 g of Triton X-100 detergent). PD-10 columns were from Pharmacia.

Buffers

The labeling buffer was 50 mmol/l $NaHCO_3$, pH 8.5, containing 9 g/l NaCl. The elution buffer was 50 mmol/l Tris-HCl, pH 7.8, containing 9 g/l NaCl and 0.5 g/l NaN_3. The coating buffer was K_2HPO_4 (50 mmol/l, pH 8.5), and the saturation solution was 50 mmol/l NaH_2PO_4, 2 H_2O, 60 g/l sorbitol and 5 g/l bovine serum albumin. The dilution buffer was 50 mmol/l Tris-HCl, pH 7.75, 9 g/l NaCl, 5.0 g/l bovine serum albumin, 0.5 g/l bovine γ-globulin, 100 mg/l Tween 40, and 20 µmol/l di-ethylene-triaminopentaacetic acid. The washing solution was 100 mmol/l Tris-HCl, pH 7.75, with 3 g/l Tween 20.

Standard

Human serum standard OKTL (Behring, Marburg, Germany), which has an apolipoprotein B concentration of 1.15 g/l, was diluted with buffer to obtain six standard concentrations.

Labeling

Purified rabbit anti-human apolipoprotein B IgG antibodies (150 µl) were loaded onto a PD 10 column rinsed with labeling buffer. Fractions of 500 µl were collected and protein absorbance was measured at 280 nm with an Ultraspec II (Pharmacia). The fraction corresponding to peak absorbance (about 2.5 ml of elution buffer) containing about 1.0 g/l IgG (an absorbance value of 1.34 corresponding to 1 g/l IgG). A volume of 250 µl (0.25 mg IgG) was added to 0.05 mg of labeling reagent (containing the europium-chelate) and incubated overnight at room temperature with gentle stirring.

Purification of europium-labeled IgG

The preparation was purified by gel filtration through a PD-10 column and eluted with elution buffer. Ten 1-ml fractions were collected and absorbance was read at 280 nm. Fractions corresponding to peak IgG absorbance were pooled.

Characterization of europium-labeled IgG

The amount of europium-labeled antibody bound to anti-apolipoprotein B was determined by measuring europium fluorescence relative to that of the europium chloride standard (100 nmol/l). After addition of enhancement solution, the fluorescence of the europium-2-naphthoyl trifluoroacetone chelate was measured as counts per second in a time-resolved fluorometer. The protein content of the europium-labeled antibody was quantified after measuring absorbance at 280 nm as:

$$\text{Protein (mol/l)} = A_{280} - (0.008 \times Eu) \times 10^6/A \times M_r$$

(A_{280}: absorbance of eluted fraction at 280 nm, 0.008: absorbance of bound aromatic thiourea, Eu: amount of europium (mmol/l), A: absorbance of IgG (1.34), M_r: relative molecular mass of IgG (160 000)).

The europium/protein ratio was used to calculate the conjugation yield (7 europium molecules per IgG molecule). The labeled antibody was stored with stabilizer to improve the stability of the europium-labeled IgG. There was no loss of immunoreactivity during six months of storage at 4 °C.

Coating of microtitre plates

Unlabeled anti-apolipoprotein B IgG was diluted in buffer to a final concentration of 100 mg/l. Polystyrene microtitre plates (Microwell™, Nunc Inc., Naperville, USA) were coated by adding 200 µl of the antibody solution per well and incubating the plates for 1 h at 37 °C. The wells were then washed three times with 250 µl of saturation solution per well to block non-specific binding sites, aspirated and used immediately.

Immunoassay procedure

Human serum standard dilutions were made in dilution buffer from 10^{-6} to 2.5×10^{-2}. Serum and CSF samples were also diluted in dilution buffer to 2.5×10^{-5} and 10^{-1}, respectively. Two hundred µl of each dilution was placed in duplicate wells and incubated for 2 h at room temperature on an automated shaking device. The reaction was stopped by washing the wells six times with washing solution, using the 12-well aspirating-washing device. Europium-labeled anti-apolipoprotein B antibody was diluted to a final concentration of 250 µg/l in assay buffer, then 200 µl was added per well and incubated for 1 h at room temperature with gentle shaking. The reaction was stopped as described above. The amount of labeled antibody bound to apolipoprotein B was quantified by dissociating europium from the antibody with enhancement solution (200 µl per well), followed by 10 min incubation with shaking and a further 10 min without shaking. The fluorescence of europium − 2-naphthoyltrifluoroacetone acetate was measured as counts per second in the time-resolved fluorometer. The instrument's data-reduction program was used to calculate the concentration of apolipoprotein B in the samples.

Criteria of reliability

Linearity

The linearity of the method was determined using the human serum standard diluted to apolipoprotein B concentrations between 1.15 and 57 500 µg/l. Each dilution was assayed six times in three separate runs.

Detection limit

The lower detection limit of the assay was defined as the concentration corresponding to the mean plus 3 standard deviations of the zero standard fluorescence signal (for 15 replicates in 5 analytical runs).

Precision

The intra-assay precision of the TR-IFMA method was studied by analysing the human serum standard at three apolipoprotein B concentrations (11.5, 115 and 575 µg/l) 20 times each. Inter-assay precision was studied by analysing control solutions at two apolipoprotein B concentrations (11.5 and 575 µg/l) 15 times each over a period of three weeks.

Intermethod comparison

A comparative study was performed with an electroimmunodiffusion assay with serum and CSF samples for patients from groups A and B, and with an immunonephelometric technique (BNA, Behringwerke, Marburg, Germany) with serum samples from normolipidaemic and dyslipidaemic subjects.

Immunoreactivity of anti-apolipoprotein B antibodies with apolipoprotein B in isolated LDL and serum

LDL of the density range of 1.03−1.05 kg/dm³ was obtained by ultracentrifugation of serum from normolipidaemic subjects (16). Isolated LDL was dialyzed against dilution buffer (pH 7.75). Apolipoprotein B in the isolated LDL fraction was then measured by immunonephelometry (apolipoprotein B: 0.9 g/l) and stored at 4 °C for 24 h. Curves of fluorescence intensities versus dilutions of isolated LDL and human serum standard (apolipoprotein B: 1.15 g/l) were constructed. Their slopes were compared to test the immunoreactivity of the anti-apolipoprotein B antibodies used in the TR-IFMA.

Comparison of dose-response curves for apolipoprotein B in serum and CSF

Dose-response curves (apolipoprotein B concentration versus fluorescence counts) were constructed for serum and CSF.

Other assays

Albumin and IgG were determined in serum and CSF by means of electroimmunodiffusion as previously described (17). Serum samples were analysed for cholesterol and triacylglycerols by enzymatic methods with a SMAC II-analyser™ (Bayer Diagnostics, Puteaux, France).

Evaluation of intrathecal synthesis

The blood/CSF ratio of apolipoprotein B was calculated. An index analogous to the CSF IgG index was calculated as follows:
Apolipoprotein B index = (CSF apolipoprotein B/serum apolipoprotein B)/(CSF albumin/serum albumin).
Local intrathecal apolipoprotein B synthesis was calculated using the formula of *Schuller* (18), as:
if CSF albumin ≤ 210 mg/l, local intrathecal apolipoprotein B synthesis = CSF apolipoprotein B − 200;
if CSF albumin > 210 mg/l, local intrathecal apolipoprotein B synthesis = CSF apolipoprotein B − (200 + ({CSF albumin − 210}/153) × (serum apolipoprotein B)). In this formula, 200 is the upper limit of normal filtration of apolipoprotein B and 153 the transudation equivalence of apolipoprotein B related to its relative molecular mass (549 000) comparatively to albumin transudation; 210 mg/l is the mean reference value of CSF albumin.

Statistical analysis

The serum apolipoprotein B concentrations obtained by TR-IFMA and immunonephelometry were compared using the *Mann* and *Whitney* non-parametric test. Intermethod comparisons were made by using the *Spearman* rank test and a regression line was plotted.

Results

Criteria of reliability

Linearity

The method was linear for apolipoprotein B concentrations between 3 µg/l and 11 500 µg/l. In standard use, the calibration curve for the human serum standard can be established at apolipoprotein B concentrations from 3 µg/l to 1150 µg/l (fig. 1).

Detection limit

The lower detection limit of the TR-IFMA for apolipoprotein B was 0.3 µg/l.

Precision

The within-run coefficients of variation (CVs) were 12.5% for 11.5 µg/l, 5.8% for 115 µg/l and 5.2% for 575 µg/l apolipoprotein B. The between-run CVs were 15.2% for 11.5 µg/l and 8.2% for 575 µg/l apolipoprotein B.

Intermethod comparison

No apolipoprotein B was detected in CSF by means of electroimmunodiffusion with a detection limit of 1 mg/l. In serum, there was good positive agreement between TR-IFMA and electroimmunodiffusion in group A and B ($r = 0.65$; $p < 0.01$). There was also good positive

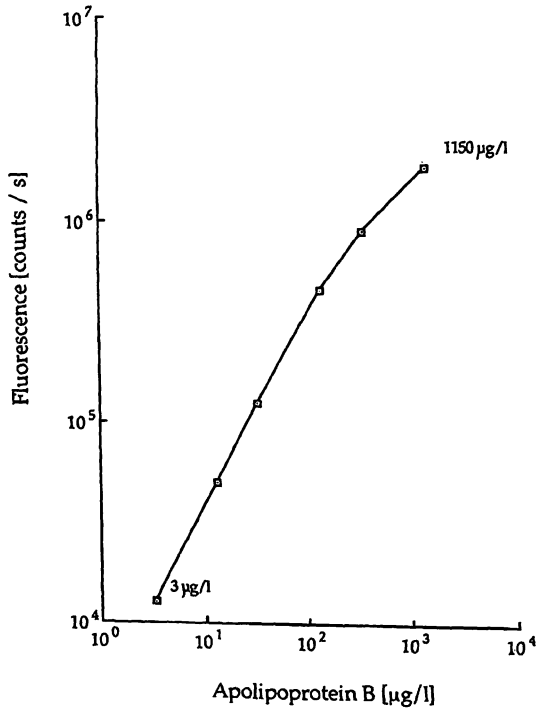

Fig. 1 Calibration curve for the measurement of apolipoprotein B by TR-IFMA – Serum standard OKTL (Behring, Marburg, Germany), apolipoprotein B: 1.15 g/l.

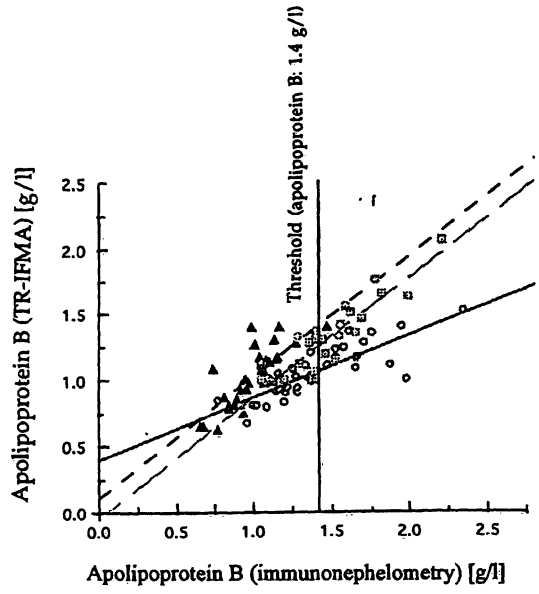

Fig. 2 Correlation between TR-IFMA and immunonephelometry for the measurement of apolipoprotein B in normolipaemic (▲) ($y = 0.93x + 0.10$; $r = 0.82$), hypercholesterolaemic (□) ($y = 0.47x + 0.40$; $r = 0.82$) and hypertriacylglycerolaemic subjects (○) ($y = 0.91x - 0.04$; $r = 0.91$) (group C).

agreement between TR-IFMA and immunonephelometric apolipoprotein B values for all the subjects in group C ($p < 0.001$) and in each subgroup (normolipidaemic, hypercholesterolaemic and hypertriacylglycerolaemic) ($p < 0.001$) (fig. 2). The mean serum apolipoprotein B concentrations in normolipidaemic subjects were 1.02 g/l and 1.01 g/l by TR-IFMA and immunonephelometry, respectively. The mean apolipoprotein B concentration in the subjects with hypercholesterolaemia was slightly lower by TR-IFMA than by immunonephelometry ($p < 0.05$) (1.27 g/l and 1.44 g/l, respectively). The TR-IFMA apolipoprotein B concentration was 15% lower than the immunonephelometric value for apolipoprotein B < 1.4 g/l and 14% lower for apolipoprotein B ≥ 1.4 g/l. The mean apolipoprotein B concentration in the subjects with hypertriacylglycerolaemia was lower by TR-IFMA than by immunonephelometry ($p < 0.001$) (1.03 g/l and 1.36 g/l, respectively). The TR-IFMA apolipoprotein B concentration were 27% lower than immunonephelometric values for apolipoprotein B < 1.4 g/l and 42% lower for apolipoprotein B ≥ 1.4 g/l.

Immunoreactivity of the anti-apolipoprotein B antibodies with apolipoprotein B in isolated LDL and serum

The slopes of the curves for fluorescence counts versus dilutions of isolated LDL and human serum standard OKTL were similar at apolipoprotein B concentrations from 225 to 9000 µg/l. The dose-response curves for

serum and CSF apolipoprotein B concentrations were identical (data not shown).

CSF and serum apolipoprotein B concentrations, apolipoprotein B blood/CSF ratio, index and local intrathecal synthesis in groups A and B

Table 1 gives the means and standard deviations for the serum and CSF apolipoprotein B and albumin concentrations and the apolipoprotein B blood/CSF ratio, index and local intrathecal synthesis in groups A and B. There was a highly significant correlation between CSF apolipoprotein B and CSF albumin ($r = 0.79$; $p < 0.001$) in group A and ($r = 0.65$; $p < 0.005$) in group B. There was also a significant correlation between CSF apolipoprotein B and albumin quotient ($r = 0.77$ in group A and $r = 0.71$ in group B; $p < 0.001$). There were no differences between the two groups in any of the quantities studied.

Discussion

Several immunoassays have been used for the measurement of apolipoprotein B in serum (19). These assays are usually not sensitive enough for the measurement of small amounts of apolipoprotein B in other body fluids such as CSF. In addition, the most suitable for routine use, immunoturbidimetry and immunonephelometry, are subject to interference by turbidity in the sample (20).

In order to measure apolipoprotein B in CSF, we developed a highly sensitive method, TR-IFMA. Reliability was satisfactory. The sensitivity of the TR-IFMA (0.06×10^{-3} µg/well) was better than that of RIA ($0.3 - 10 \times 10^{-3}$ µg/well) and ELISA ($0.1 - 0.5 \times 10^{-3}$ µg/well) (21), owing to both the retained immunoreactivity of the chelate-labelled antibodies and the highly sensitive time-resolved fluorescent detection method for enhanced chelate fluorescence (22). The working range was large, contrary to ELISA (11), and within- and between-run precision was good. A correlation was obtained between TR-IFMA and electroimmunodiffusion in serum and immunonephelometry for serum apolipoprotein B concentrations in group C. The mean apolipoprotein B concentrations obtained with TR-IFMA matched those obtained by immunonephelometry in the normolipidaemic and hypercholesterolaemic subgroups. The higher values obtained by immunonephelometry in the hypertriacylglycerolaemic subgroup could be related to the difference observed between one-site (nephelometry) and two-site (TR-IFMA) assays (23). These higher values could be due to the sensitivity of the nephelometric method to the size of the analyte, owing to differences in light scattering (24). Indeed, the measurement of apolipoprotein B can be inaccurate in samples containing large triacylglycerol-rich particles (12). The overestimation of apolipoprotein B by immunonephelometry in hypertriacylglycerolaemic serum is overcome by TR-IFMA which, by measuring fluorescence, is independent of sample turbidity (25).

The equal immunoreactivity of the anti-apolipoprotein B antibodies with apolipoprotein B in different forms (serum and isolated LDL), and the equal dose-response curves for serum and CSF apolipoprotein B, makes this method well adapted to the quantification of apolipoprotein B in CSF.

The sensitivity of TR-IFMA was sufficient to detect small amounts of apolipoprotein B in CSF from all the patients studied. No apolipoprotein B was detected in CSF by electroimmunodiffusion (detection limit: 1 mg/l). These observations are in agreement with reports by *Roheim* et al. (3) and *Pitas* et al. (1). Using a sensitive ELISA, *Carlson* et al. also reported the presence of apolipoprotein B in CSF (4). The higher CSF apolipoprotein B concentrations described by *Carlson* (0.77 ± 3.4 mg/l versus 0.12 ± 0.06 mg/l in our study) could be explained by difference in the patients studied. Indeed, these authors studied only neurological patients with large variations in CSF albumin, i. e. with blood-CSF barrier damage. No differences were observed in serum and in CSF apolipoprotein B concentrations between controls and multiple sclerosis patients, none of whom had increased blood-CSF barrier permeability. As *Carlson* reported, CSF apolipoprotein B concentrations were several orders of magnitude lower than the corresponding serum concentrations: the blood/CSF ratios

Tab. 1 Concentrations of quantities studied in control subjects (group A) and multiple sclerosis patients (group B)

	Serum apolipoprotein B (mg/l)		CSF apolipoprotein B (mg/l)			
	TR-IFMA	EID	TR-IFMA	EID	Blood/CSF ratio	Index
Group A	934 ± 180*	1149 ± 298	0.11 ± 0.06	<1	5920 ± 1751	0.31 ± 0.19
Group B	886 ± 219	1387 ± 456	0.12 ± 0.06	<1	6928 ± 3205	0.34 ± 0.21

* Mean ± standard deviation

were about 6000 in both controls and multiple sclerosis patients. As presumed from these very high blood/CSF ratios, no apolipoprotein B intrathecal synthesis was found by determination of both apolipoprotein B index and local intrathecal synthesis in either group A or B. Moreover, the CSF apolipoprotein B concentrations showed a good correlation with the albumin quotient and the albumin level in CSF, indicating that the small amounts of apolipoprotein B in CSF are presumably of serum origin. It is noteworthy the *Blue* et al. found no apolipoprotein B synthesis in the rooster brain (26).

The excellent reliability of this technique, particularly its high sensitivity, makes it suitable for the measurement of apolipoprotein B in CSF and other fluids containing very low concentrations of apolipoprotein B. Moreover, a very small volume of CSF (20 µl) is required. The very small amounts of apolipoprotein B in CSF indicate its probable serum origin, while the lack of differences between the controls and multiple sclerosis patients calls into question the role of this apolipoprotein in lipid transport in the central nervous system.

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